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Pesticide removal by the bioaugmentation process in secondary treated wastewater

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ABSTRACT

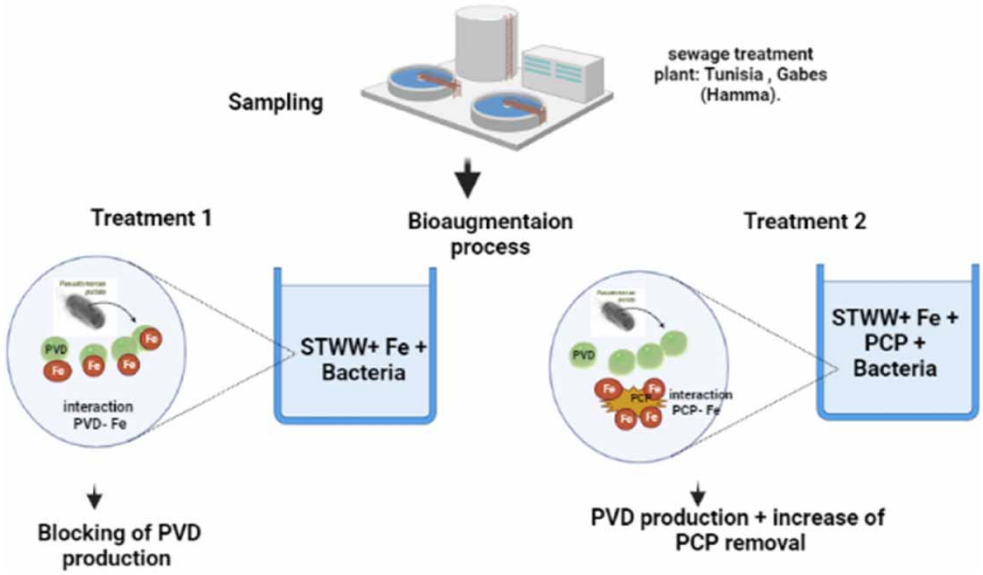
The work was focused on the effect of the bioaugmentation process on STWW contaminated by pentachlorophenol (PCP: 100 mg L^{-1}) by *Pseudomonas putida* AE015451. The monitoring of bioaugmentation treatments was assessed by chloride content determination via high-performance liquid chromatography (HPLC), optical density (OD) for microbial biomass determination, and pyoverdine and biofilm production. The process of bioaugmentation by a PGPR Pseudomonas strain showed a high-efficiency removal rate of PCP (100 mg L^{-1}). The contaminant decreased up to 92% after 168 h. The production of pyoverdine and the formation of bacterial biofilm by the strain *Ps. putida* AE015451 showed an important role in tolerating the toxicity of PCP by using it as a carbon source. The obtained result proved that the pyoverdine production and biofilm formation help the *Pseudomonas* bacteria to tolerate to the stressed condition as pesticide. Moreover, the co-existence of the iron and PCP molecule ameliorate its biodegradation.

Key words: bioaugmentation, biofilm formation, iron chelating, pentachlorophenol, PGPR strain, pyoverdine release

HIGHLIGHTS

- The bioaugmentation of STWW is an efficient alternative to the conventional methods.
- After 72 h, 90% of PCP were degraded by the action of *Ps. putida* AE015451.
- The degradation process was enhanced by the pyoverdine production and Fe addition.
- Biofilm production enhanced the resistance to PCP and then the degradative process.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Pentachlorophenol (PCP), extensively used as a herbicide, insecticide, fungicide, wood preservative, resin, lubricant, and dye intermediate, has been commonly found in groundwater, sediment, and surface soils (Lemke *et al.* 2021; Mundeja *et al.* 2021). The World Health Organization (1996) reports that the concentration of PCP in industrial wastewater ranged between 2 and 50 mg L^{-1}, the concentration in rivers is 10.5 mg L^{-1}, and the concentration in water samples is 0.01 mg L^{-1}. Since PCP is a toxic substance with high mutagenicity and carcinogenicity, researchers have considered removing it from industrial wastewater (Seyedi *et al.* 2019). PCP can be degraded in the environment by chemical (Asgari *et al.* 2021), microbiological (Werheni Ammeri *et al.* 2021a, 2021b, 2022a, 2022b, 2022c, 2022d), and photochemical processes (Cabezuelo *et al.* 2021). Many strains of bacteria and fungi, such as *Sphingomonas chlorophene* (Yang *et al.* 2006), *Acinetobacter* ISTPCP-3 (Sharma *et al.* 2009), and *Sphingobium chlorophenolicum* ATCC 39723 (Dams *et al.* 2011), have been shown to have PCP degradation capabilities. The transformation of PCP in an aqueous solution led to the release of chlorine, its quantification showed the PCP degradation efficiency (Asgari *et al.* 2021). Members of the genus *Pseudomonas* showed remarkable metabolic and physiological versatility, allowing the colonization of various terrestrial, and aquatic habitats and tolerance against many xenobiotics (Hosu *et al.* 2021), and are of great interest because of their increasing potential in biotechnological (Hassen *et al.* 2021; Raio & Glimcher 2021). Iron is an essential nutrient for most living organism to sustain their growth, although it is mostly unavailable in the environment. Researchers report that the iron cycle can be combined with the carbon cycle, the fate of heavy metals, and the conversion of nitrogen and organic pollutants (Li *et al.* 2012; Yu *et al.* 2013). Recently, it was observed that PCP can be chemically dechlorinated by the high reaction activity of adsorbed Fe (II) produced from the dissimilatory iron reduction process mediated by iron-reducing microorganisms (Wang *et al.* 2017). Siderophores, produced by a different class of microorganisms (bacteria, fungi, etc.), are small molecules with a high affinity for Fe (III) and can be produced in iron-limited conditions (Neilands 1981). Therefore, many strategies have been developed by organisms such as fungi, plants, or bacteria to have access to this element essential for their growth (David *et al.* 2019). Several *Pseudomonas* species have the capacity to supply and excrete, below iron-restricting conditions, soluble yellow-green fluorescence pigments (Bultreys *et al.* 2003) named pyoverdines (PVD) or pseudobactins, which act as siderophores for those bacteria (Meyer 2000). These molecules are idea to be related to pathogenesis (Fuchs *et al.* 2001). Bacteria can come to be tolerant to oxidative pressure with the aid of using secreting extracellular polymeric substances and forming biofilm (Rovida *et al.* 2021). The bacterial flair of colonizing a poisonous biotic floor boomed with biofilm development. The environmental pressure situations may want to affect biofilm production (Poole 2014) and represent a unique mode of increase that lets for survival in adverse environments (Elias & Banin 2014). Meliani & Ben Soltane (2014) stated that Pseudomonas isolates broaden an essential

biofilm mass development, to defend cells from adverse environments. The microorganisms that make up biofilms may be used of polluting substances as a supply of carbon and energy (Cohen *et al.* 2002; Petrova & Sauer 2012).

The aim of this study was to check the capability of the Plant Growth-Promoting Rhizobacteria (PGPR) strain *Ps. putida* AE015451 to tolerate and remove the pesticide PCP in liquid MSM or treated secondary wastewater (TSWW). We follow the effect of the PCP during the pyoverdine secretion (PVD) by the *Ps. putida* AE015451. The biofilm formation capacity of this strain was studied under stressed conditions following PCP and Fe addition. The effect of PCP–Fe interaction on the production of PVD and biofilm development by *Ps. putida* AE015451 was also observed.

The novelty in our work is the study of the capacity of the bacterial strain *Ps. putida* AE015451 in the elimination of PCP in secondary wastewater. Also, the effect of Fe addition in the improvement of PCP biodegradation. Thus, the effect of Fe–PCP interaction in the production of pyoverdine-type siderophore. The main study's findings showed us how effective the *Pseudomonas* bacteria are in removing harmful pesticides.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents reagent-grade

PCP (MW = 266,337 > 99% purity) was purchased from Sigma-Aldrich (USA) and high-performance liquid chromatography (HPLC) grade solvents were purchased from Merck, Germany. All chemicals used for the culture media preparation and other reagents used were purchased from Sigma-Aldrich or Fluka.

2.2. Wastewater sampling and determination of the main physical–chemical characteristics

Wastewater with no detectable PCP was sampled in April 2021 at the level of Gabes El Hamma wastewater treatment plant in the arid region of southern Tunisia. The sewage treatment plant has a secondary wastewater treatment system (STWW) with activated sludge. STWW samples were stored at 4 °C to determine key physical and chemical properties: cation exchange capacity (CEC) and pH, organic carbon, chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), nitrate, chloride, total nitrogen, and total carbon (Brook *et al.* 1971; Werheni Ammeri *et al.* 2023).

2.3. Bacterial strain selection

The *Ps. putida* strain AE015451 used in this work was isolated from an industrial wastewater plant and analyzed by partial 16S rRNA gene sequencing by Mehri *et al.* (2011). The strain was selected by testing its resistance and ability to eliminate PCP in MSM at a concentration of 30–300 mg L^{-1} for 168 h (Mundeja *et al.* 2021). The composition of MSM was in mg L^{-1}: KH_2PO_4, 800; Na_2HPO_4, 800; $MgSO_4{\cdot}7H_2O$, 200; $CaCl_2{\cdot}2H_2O$, 10; NH_4Cl, 500; and 1 mL of trace metal solution comprising in (mg L^{-1}) $FeSO_4{\cdot}7H_2O$, 5; $ZnSO_4{\cdot}H_2O$, 4; $MnSO_4{\cdot}4H_2O$, 0.2; $NiCl{\cdot}6H_2O$, 0.1; H_3BO_3, 0.1; $CoCl_2{\cdot}6H_2O$, 0.5; $ZnCl_2$, 0.25; EDTA, 2.5 with a pH 6.3. PCP concentration was added to the medium after autoclaving. The flasks were incubated at 30 °C under constant shaking at 160 rpm min^{-1} using an incubator shaker (ZHWY-2102 P). All the treatments were carried out in triplicate.

2.4. Bioaugmentation experiments

The preparation of *Ps. putida* AE015451 inoculum for the bioaugmentation process study was made into the nutrient broth for 24 h at 30 °C. The obtained inoculum was centrifuged for 10 min at 12,000 rpm and 4 °C. The test of PCP removal capacity was described by Werheni Ammeri *et al.* (2021) and was released in MSM and sterile STWW. The choice of MSM liquid medium is specific to *Pseudomonas* bacteria according to the literature (Karn *et al.* 2010a; Werheni Ammeri *et al.* 2017, 2021a, 2021b) and in addition contains only PCP as a carbon source. The STWW was sterile after three autoclaves at 120 °C for 15 min. A variation in different parameters has been considered: variation of inoculum volume (0.5, 1, and 2 mL), PCP (30, 50, 70, 100, 200, and 300 mg L^{-1}), iron concentration (5, 10, and 20 mg L^{-1}), and pH (4, 5, 6.3, 7, or 8). The experiment was performed in Erlenmeyer flasks containing 100 mL of MSM or sterile STWW. Samples for measurement were taken to the initial time of incubation (T0) and after 168 h of incubation (TF). The experiment was carried out in triplicates.

2.4.1. Bacterial biomass determination

The microbial biomass values of the *Ps. putida* AE015451 strain used in this study were measured by a spectrometer (UV–Vis Dual BEAM UVS-2700) at 600 nm and at 24 h intervals to measure the OD of the samples. The bacterial biomass was estimated according to the formula, Bacterial biomass = OD/k, with k the number of bacteria counted on MSM agar in 1 mL of inoculum (Cabezuelo *et al.* 2021).

2.4.2. PCP content determination

The study of PCP removal was quantified by HPLC analysis (Rao *et al.* 2017; Werheni Ammeri *et al.* 2022b). The PCP was extracted by methanol solution, and a volume of 1 mL of the different treatments taken at 24 h incubation intervals. The prepared suspension was vortexed for 5 min and could stand at 20 °C for 10 min. After, the sample was vortexed for 5 min and centrifuged at 8,000 rpm for 5 min, and the supernatant was filtered through a 0.22 μm sterile filter. The filtrate was analyzed by a Perkin Elmer Series YL9100 HPLC as reported by Karn *et al.* (2010a). All analyzes were carried out in triplicates.

2.4.3. Chloride content determination

The chloride content in MSM and STWW for different treatments in the bioaugmentation process was determined in a neutral medium using a silver nitrate titration method in the presence of potassium chromate described by Werheni Ammeri *et al.* (2021a).

2.4.4. Pyoverdine determination

Deficient iron Casamino-acid liquid culture medium (CAA) is used in this study and comprises 5 g of casamino-acid, 1.18 g of K_2HPO_4 and 0.25 g of $MgSO_4$ in 1 L of deionized water. The sterile medium CAA was inoculated with the strain *Ps. putida* AE015451 and incubated for 48 h at 25 °C as described by Mehri *et al.* (2011). Different treatments were performed in this experiment depending on the volume inoculum variation, the concentration of PCP (30, 50, and 100 mg L^{-1}) and at different pH values varying between 5 and 8. The amount of pyoverdine extracted from the culture medium was spectrophotometry determined at 400 nm according to the method described by Navazio (2005) and David *et al.* (2020).

2.4.5. Biofilm quantification by the Polystyrene Microplate Test

This biofilm formation protocol was adapted from methods used by Merritt *et al.* (2003) and Malcova *et al.* (2008). Bacterial strains were incubated in heart-brain broth (BHI: MAST DM106) and MSM with 0.25% glucose for 24 h at 30 °C (Werheni Ammeri *et al.* 2022d). The growing bacterial culture was transferred to a 96-well microtiter plate. Microtiter plate tests were performed in triplicate, in triplicate per well/treatment (PCP or no and Fe) along with negative controls. Calculate OD by subtracting the mean of 3-fold OD from ODC (control). Absorbance was measured at 550 nm using an automated system (UV–Vis Dual BEAM UVS-2700). Strains were classified as low, medium, or high biofilm producers based on OD (Mehri *et al.* 2014; Werheni Ammeri *et al.* 2021a, 2021b).

2.5. Statistical analysis

Statistical analysis was performed using the SPSS 21.0 software. Data collected, PCP removal %, chloride and bacterial biomass content of MSM and STWW samples, were subjected to analysis of variance (ANOVA), and the means were separated by the Duncan test at $P \leq 0.05$. Differences between the two sampling times were compared by an independent-sample *t*-test (two-tailed). All data were presented as mean ± SD ($n = 3$). The relationship between the parameters was carried out by the Minitab software to generate the specific response and the contour plot.

3. RESULTS AND DISCUSSION

3.1. Wastewater physical and chemical characteristics

The results obtained from the characterization of the STWW sample used in this study are presented in Table 1. The STWW sample has a basic pH of 8.7 and a high COD and BOD_5 of 632.0 and 380 mg L^{-1}, respectively. According to the COD/BOD_5 ratio lower than 2, the studied effluent is easily biodegradable (Płuciennik-Koropczuk & Myszograj 2019). However, the COD recorded for our sample is lower than textile wastewater according to the work of Yakamercan & Aygün (2020). Thus, the chloride level in STWW is 14.6 g L^{-1}. The nitrogen value obtained from the STWW showed a value of 48.0 mg L^{-1}. The STWW showed a higher chloride value as compared with a preliminary study conducted Werheni Ammeri *et al.* (2021). The growth of *Ps. putida* in the STWW sample was rather difficult.

3.2. Effectiveness of *Ps. putida* AE015451 in the PCP bioaugmentation essay

The growth curve of *Ps. putida* AE015451 in MSM and STWW medium supplemented with PCP showed its tolerance capacity toward this pollutant (Figure 1). In the MSM, the bacterial growth followed a decrease until 48 h of incubation time from 7.82 to 7.10 log CFU mL^{-1}, instead in the STWW sample an exponential phase until 48 h was observed 6.90–8.78 log CFU mL^{-1} (Figure 1(a)). In addition, during this phase, the PCP removal percentage was 10.32 and 24.23%, in MSM and

Table 1 | Physical and chemical characteristics of the secondary wastewater (STWW) sample (data are represented as Means ± SD, $n = 3$)

Parameter	Value
Dry matter, %	38.0 ± 1.0
pH	8.7 ± 0.9
Conductivity, mS cm^{-1}	1.8 ± 0.1
Organic carbon, %	2.1 ± 0.1
Nitrogen, mg L^{-1}	48.0 ± 1.0
COD, mg L^{-1}	632.0 ± 4.0
BOD_5, mg L^{-1}	380 ± 0.2
Nitrates, mg L^{-1}	2.3 ± 0.1
Chlorides, g L^{-1}	14.6 ± 0.5

COD, chemical oxygen demand; BOD_5, biochemical oxygen demand.

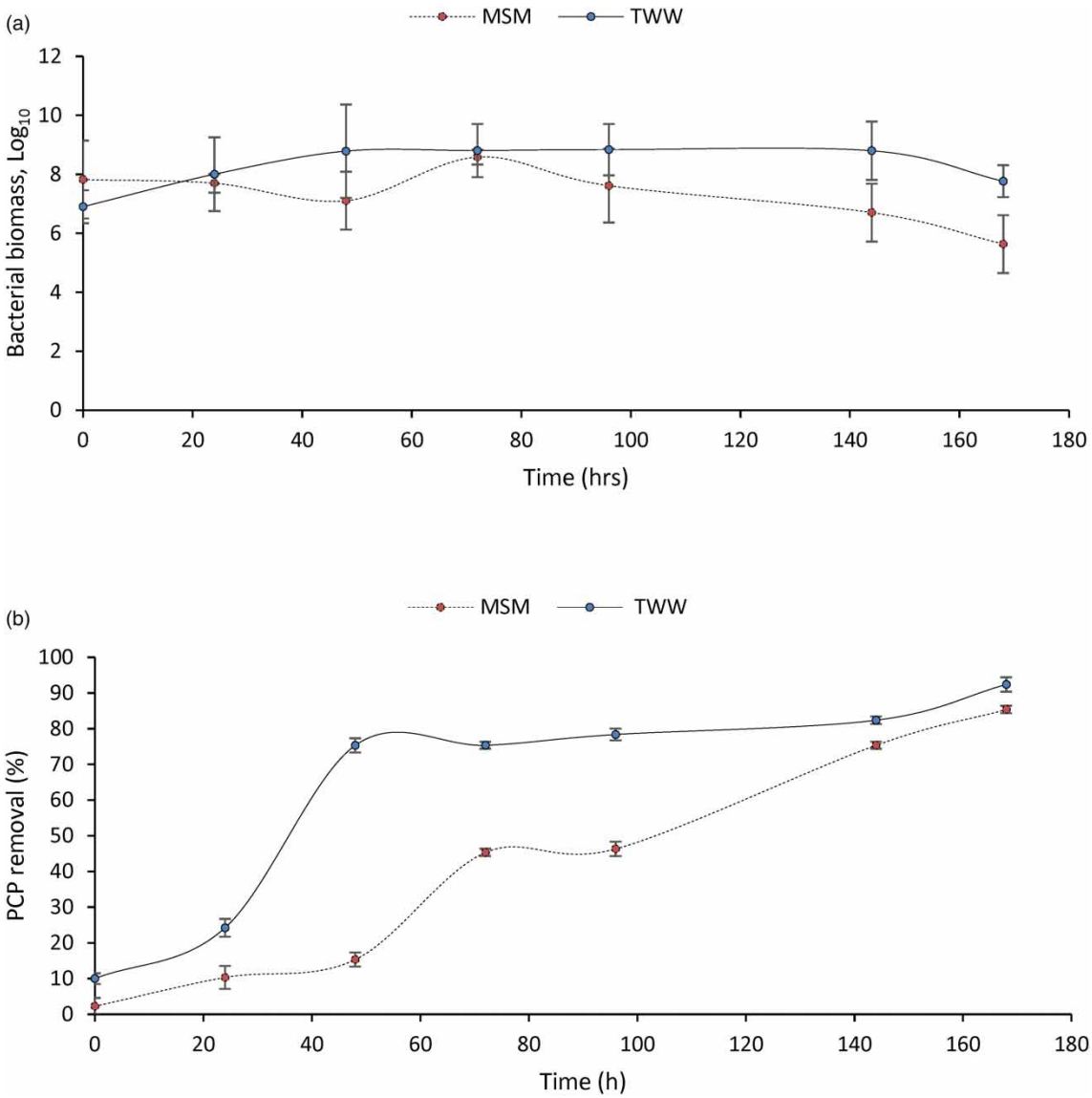


Figure 1 | Bacterial biomass variation (a) and PCP (100 mg L^{-1}) removal% (b) in the mineral medium (MSM) and secondary wastewater (STWW) with 1 mL of inoculum after 168 h.

STWW samples, respectively (Figure 1(b)). These obtained results could be explained by the potential of enzymatic activity that the bacteria are about to multiply especially in STWW samples. The bacterial biomass increased to 7.61 log CFU mL^{-1} in the MSM sample (96 h), while a lag phase was observed until 144 h in STWW samples (8.80 log CFU mL^{-1}). The MSM sample followed a decrease of the bacterial growth after 96 h, reaching 5.63 log CFU mL^{-1} at the end of the incubation 168 h. The PCP removal exponentially increases after 48 h in STWW samples by reaching a 75.33% of removal percentage. In the same sample, the percent of PCP removal further increase to 92.36% after 168 h of incubation time. In the MSM sample, the PCP removal rate increased until the end of the incubation time by reaching 85.36% removal. So, different behaviors of bacterial biomass and PCP removal in MSM and STWW samples were observed. The greater PCP removal was achieved during the exponential phase of the bacterial growth. Finally, the microbial growth in the MSM and STWW samples enters the phase of decline after 144 h of incubation because of the death of bacteria in MSM and STWW. These results confirm the efficiency of *Ps. putida* AE015451 bacteria to remove PCP from secondary wastewater.

In this study, the selection of a strain of *Ps. putida* AE015451 species was based on its ability to eliminate PCP molecules (Werheni Ammeri *et al.* 2022a, 2022b, 2022c, 2022d). As reported by Lee *et al.* (2011), the strain *Pseudomonas* sp. (Bu34) can remove the rate of 4,000 mg L^{-1} PCP. Also, Karn *et al.* (2010a) proved that *Ps. stutzeri* CL7 can grow up in the PCP contaminated medium (600 mg L^{-1}). In our previous study Werheni Ammeri *et al.* (2017), we demonstrated that *Ps. fuorescens* can tolerate and remove up to 250 mg L^{-1} of PCP in the MSM medium after 96 h of incubation at controlled conditions.

3.3. Chloride content variation

The chloride content in the control sample (MSM or STWW + *Ps. putida* AE015451) significantly increased at TF compared to T0 (Table 2). Different experimental conditions were studied such as PCP, Fe content, inoculum volume, and pH of the matrix. Karn *et al.* (2010a) proved that the PCP used and the discharge of chloride with inside the medium was essential because of the PCP mineralization.

3.3.1. PCP content

Chloride content was determined for the different PCP concentrations (30, 50, 70, 100, 200, and 300 mg L^{-1}). Different behaviors of the chloride content were observed in MSM or STWW samples (Table 2). A significant decrease in chloride content

Table 2 | Chloride concentration (g L^{-1}) at T0 and after 7 days (TF) in the bioaugmentation experiment with *Pseudomonas putida* AE015451 in MSM and STWW samples under different experimental conditions

		MSM		STWW	
Treatments		**T0**	**TF**	**T0**	**TF**
Control	–	0.56 ± 0.01 b	0.80 ± 0.02 a	1.25 ± 0.36 b	3.60 ± 0.35 a
PCP (mg L^{-1})	30	1.36 ± 0.03 Db	4.20 ± 0.03 Aa	2.05 ± 0.17 Cb	3.40 ± 1.04 Ca
	50	1.98 ± 0.01 Ab	3.80 ± 0.03 Ba	2.05 ± 0.16 Cb	3.20 ± 0.45 Ca
	70	1.69 ± 0.06 Bb	2.80 ± 0.46 Ca	1.07 ± 0.36 Db	3.40 ± 0.54 Ca
	100	1.33 ± 0.04 Db	1.80 ± 0.33 Da	2.36 ± 1.03 Cb	4.20 ± 0.33 Ba
	200	1.50 ± 0.07 Ca	1.60 ± 1.03 Da	5.24 ± 3.03 Bb	14.20 ± 0.25 Aa
	300	1.51 ± 0.05 Ca	1.68 ± 0.99 Da	8.33 ± 2.04 Ab	14.00 ± 0.33 Aa
Fe (mg L^{-1}) + 30 mg L^{-1} PCP	5	0.40 ± 0.03 Bb	2.35 ± 0.01 Aa	2.31 ± 0.21 Ab	3.80 ± 0.03 Aa
	10	0.52 ± 0.01 Ab	2.30 ± 0.04 Aa	2.01 ± 0.36 Ab	3.00 ± 0.15 Ba
	20	0.48 ± 0.03 Ab	1.95 ± 0.01 Ba	1.98 ± 0.33 Ab	3.00 ± 0.28 Ba
Inoculum (mL) + 30 mg L^{-1} PCP	0.5	0.46 ± 0.01 Bb	6.20 ± 0.03 Aa	1.52 ± 0.98 Aa	2.20 ± 0.03 Ba
	1	0.65 ± 0.03 Ab	8.40 ± 0.03 Ba	1.52 ± 0.65 Aa	4.40 ± 1.24 Aa
	2	0.37 ± 0.04 Cb	4.60 ± 0.04 Ca	2.01 ± 0.14 Ab	2.40 ± 1.10 Ba
pH + 30 mg L^{-1} PCP	4	0.89 ± 0.06 Db	1.12 ± 0.66 Ba	2.01 ± 0.66 Ab	3.80 ± 0.33 Aa
	5	0.79 ± 0.06 Db	3.40 ± 0.99 Aa	2.04 ± 0.14 Ab	2.40 ± 0.43 Aa
	6.3	1.02 ± 0.04 Cb	3.20 ± 0.53 Aa	0.80 ± 0.25 Bb	2.72 ± 0.43 Ba
	7	1.36 ± 0.04 Bb	2.90 ± 0.04 Aa	0.98 ± 0.33 Bb	2.20 ± 0.53 Ba
	8	1.45 ± 0.02 Ab	3.26 ± 0.02 Aa	1.04 ± 0.99 Bb	2.40 ± 0.44 Ba

Different capital letters show significant differences among the different experimental conditions (Fe concentration, Inoculum volume, PCP concentration, and pH) in the sampling time. The different lowercase letter shows differences between the two sampling times (T0 and TF).

at T0 and TF was observed by increasing PCP concentration, from 1.98 ± 0.03 g L^{-1} of the 50 mg L^{-1} to 1.33 ± 0.04 g L^{-1} of the 100 mg L^{-1} PCP contaminated MSM samples of T0 time and from 4.20 ± 0.03 to 1.60 ± 1.03 g L^{-1} of the 30 and 200 mg L^{-1} PCP contaminated MSM samples of TF time (Table 2). In addition, a significant increase in the chloride content was observed from T0 to TF in the 30–100 mg L^{-1} PCP contaminated MSM samples, by increasing the PCP to 200 and 300 mg L^{-1} not significantly changes from T0 and TF, were observed (Table 2). Contrariwise, in the STWW sample by increasing the PCP concentration, a significant increase of the chloride content was observed by reaching 8.33 ± 2.04 and 14.20 g L^{-1} at T0 and TF in the 300 and 200 mg L^{-1} of PCP (Table 2). Furthermore, a significant increase from T0 to TF in the different PCP contaminated STWW samples was observed (Table 2). Using PCP as the sole carbon source led to a release of chlorides in the medium. These results agreed with the study of Werheni Ammeri *et al.* (2021), in which *Ps. putida* strain can remove PCP molecule 800 mg L^{-1} and release proportional rates of chloride.

3.3.2. Fe content

The effect of iron rate variations (5, 10, and 20 mg L^{-1}) on the chloride content in MSM and STWW supplemented with PCP 30 mg L^{-1} is represented in Table 2. In both liquids tested, the release of chloride decreased by increasing Fe concentration and this result could be explained by the decrease of PCP degradation. A significant increase in chloride concentration from T0 to TF was observed (Table 2). Removal of PCP by *Ps. putida* AE015451 in the presence of an increasing concentration of iron (5, 10, and 20 mg L^{-1}) corresponds to a decrease in chloride content in the MSM and STWW media. According to the results in both liquid media, the chloride level appeared proportional to the increase of Fe concentrations 5, 10, and 20 mg L^{-1}. When the experiment ended, the chloride level at these latter concentrations of Fe was 2.35, 2.30, and 1.95 g L^{-1} in MSM and 3.8, 3.0, and 3.0 g L^{-1} in STWW. These results could be explained by the ability of *Ps. putida* AE015451 to degrade PCP even at a high rate (300 mg L^{-1}). Similarly, in STWW, PCP appeared proportional to the release of chloride, but this release was greater by using a $FeSO_4$ rate of 5 mg L^{-1}.

Thus, PCP being chelated and the ionic transfer was enhanced by iron in the biotope, which facilitates the development of bacteria (Chen *et al.* 2011; Lin *et al.* 2014). Thus, since iron is an essential element for the metabolism of aerobic microorganisms, bacteria can also assimilate iron and grow (Beasley *et al.* 2019). Besides, the Fe addition changes the pH to acid (Martin *et al.* 2008).

3.3.3. Inoculum volume

The effect of inoculum volume (0.5, 1, and 2 mL) in the presence of 30 mg L^{-1} of PCP contaminated MSM or STWW samples is reported in Table 2. In MSM at T0 by increasing the inoculum volume, a significant decrease to 0.37 ± 0.04 g L^{-1} of the chloride content was observed. At TF, the chloride content significantly increased compared with T0 in all the treatment and within the TF time a significant decrease from 6.20 ± 0.03 to 4.60 ± 0.04 g L^{-1} was observed by increasing the inoculum volume from 0.5 to 2 mL. In the STWW sample at T0, no significant change was observed in the chloride content (Table 2), and at TF, in which no further changes were observed compared with T0, the greater value of the chloride content was registered in the sample with 1 mL of inoculum (Table 2).

3.3.4. pH variation

The pH of the medium is typically regarded as a crucial factor in PCP elimination (Karn *et al.* 2010a, 2010b). In this study, the variation of the pH between 4, 5, 6.3, 7, and 8 in 30 mg L^{-1} of PCP contaminated MSM and STWW samples is reported in Table 2. The increase of the pH in the MSM sample allowed a significant increase of the chloride content in both T0 and TF (Table 2). But in STWW samples, the chloride content showed a decrease from 2.01 ± 0.66 to 1.04 ± 0.99 g L^{-1} and from 3.80 ± 0.33 to 2.40 ± 0.44 g L^{-1} in T0 and TF, respectively (Table 2). In both MSM and STWW samples, a chloride content increase in T0 to TF was observed. The pH of the media played an important role in the PCP mineralization by microbial biomass, as reported by Cabezuelo *et al.* (2021) and Karn *et al.* (2010a, 2010b). Thus, the pH ranging between 6.5 and 8 enhanced the PCP biodegradation by augmenting its solubility and availability in the medium (Hechmi *et al.* 2013).

3.4. Bacterial biomass variation

The bacterial biomass in the control sample MSM or STWW + *Ps. putida* AE015451 showed an increase between T0 and TF (Table 3). Also, different experimental conditions were examined such as PCP, Fe content, bacterial inoculum volume, and pH of the medium.

Table 3 | Bacterial biomass (log_{10} CFU mL^{-1}) at T0 and after 7 days (TF) in the bioaugmentation experiment with *Pseudomonas putida* AE015451 in MSM and STWW samples at different experimental conditions

		MSM		STWW	
Treatments		**T0**	**TF**	**T0**	**TF**
Control	–	8.02 ± 0.04 b	8.83 ± 0.33 a	7.70 ± 0.33 b	8.94 ± 0.37 a
PCP (mg L^{-1})	30	2.24 ± 0.05 Ab	8.64 ± 0.98 Aa	7.90 ± 0.13 Aa	7.85 ± 0.15 Ba
	50	2.61 ± 0.98 Ab	7.67 ± 1.09 Aa	8.00 ± 0.25 Aa	8.64 ± 0.33 Aa
	70	2.89 ± 1.03 Ab	6.32 ± 1.92 Aa	7.80 ± 1.03 Aa	7.96 ± 0.14 Ba
	100	2.20 ± 0.93 Ab	5.51 ± 1.10 ABa	8.10 ± 0.98 Aa	8.48 ± 0.45 Aa
	200	2.23 ± 0.92 Ab	4.55 ± 1.09 Ba	7.54 ± 0.48 Aa	8.27 ± 0.33 ABa
	300	2.14 ± 1.03 Ab	4.20 ± 1.10 Ba	7.70 ± 0.66 Aa	8.55 ± 0.33 Aa
Fe (mg L^{-1}) + 30 mg L^{-1} PCP	5	8.61 ± 0.01 Aa	8.86 ± 0.47 Aa	7.70 ± 0.33 Aa	8.21 ± 0.29 Ba
	10	8.41 ± 0.04 Ba	8.79 ± 0.25 Aa	7.80 ± 0.13 Aa	7.83 ± 0.46 Ba
	20	8.37 ± 0.01 Ba	8.38 ± 0.98 Aa	8.20 ± 0.36 Ab	8.61 ± 0.15 Aa
Inoculum (mL) + 30 mg L^{-1} PCP	0.5	6.90 ± 0.02 Cb	7.76 ± 0.02 Ca	7.70 ± 0.15 Ab	8.45 ± 0.16 Aa
	1	7.09 ± 0.02 Bb	8.98 ± 0.01 Aa	7.80 ± 1.03 Aa	7.63 ± 0.46 Ba
	2	8.02 ± 0.05 Aa	8.06 ± 1.98 Aa	8.10 ± 0.98 Aa	8.66 ± 0.27 Aa
pH + 30 mg L^{-1} PCP	4	3.66 ± 0.92 Bb	5.65 ± 1.03 Ca	7.70 ± 0.33 Aa	7.54 ± 0.58 Aa
	5	3.01 ± 1.09 Bb	8.70 ± 0.92 Aa	7.80 ± 0.24 Aa	8.70 ± 0.70 Aa
	6.3	3.06 ± 1.02 Bb	9.24 ± 0.35 Aa	7.70 ± 1.04 Aa	8.83 ± 1.25 Aa
	7	7.72 ± 1.04 Aa	6.80 ± 0.78 Bb	7.60 ± 0.97 Aa	7.80 ± 0.98 Aa
	8	7.91 ± 1.03 Aa	4.22 ± 0.98 Cb	8.09 ± 0.20 Aa	8.17 ± 1.10 Aa

Different capital letters show significant differences among the different experimental conditions (Fe concentration, inoculum volume, PCP concentration, and pH) in the sampling time. The different lowercase letter shows differences between the two sampling times (T0 and TF).

3.4.1. PCP content

Table 3 presents the results about the effect of PCP content variations of 30, 50, 100, 200, and 300 mg L^{-1} on bacterial biomass (BBM) developed in the MSM medium and STWW at T0 and after 7 days (TF) of incubation at 30 °C. In MSM and STWW samples at T0, no significant changes were observed in BBM (Table 3), while, in MSM, at TF, a significant increase of the BBM was observed in all the PCP contaminated MSM (Table 3). Within TF time, the BBM value decreased by increasing the PCP concentration and went from 8.64 ± 0.98 log_{10} CFU mL^{-1} of 30 mg L^{-1} to 4.20 ± 1.10 log_{10} CFU mL^{-1} of 300 mg L^{-1} PCP contaminated MSM medium (Table 3). In STWW samples at TF, no further increase in terms of BBM was observed compared to T0 (Table 3). Contrariwise to what happens in the MSM medium at TF in the STWW sample, the BBM significantly increased from 7.85 ± 0.15 log_{10} CFU mL^{-1} of 30 mg L^{-1} to 8.55 ± 0.33 log_{10} CFU mL^{-1} of 300 mg L^{-1} PCP contaminated samples (Table 3).

Based on these results, this pesticide acts directly on the *Ps. putida* AE015451 growth; thus, bioaugmentation efficiency decreases. Therefore, the biodegradation of PCP decreases with its increasing rates (Werheni Ammeri *et al.* 2016) especially in the PCP contaminated MSM medium. In addition, the PCP has a negative effect on bacteria growth (Urrutia *et al.* 2013; Gałązka *et al.* 2018).

3.4.2. Fe content

Table 3 shows the effect of the different rates of iron in MSM and TSWW on BBM at a PCP rate of 30 mg L^{-1}. Adding the different concentrations of iron ($FeSO_4$) at 5, 10, and 20 mg L^{-1} led to a different behavior in MSM and STWW medium after 7 days (Table 3). At T0 in the MSM medium, a significant slight decrease of the BBM value was observed by increasing the Fe concentration. In addition, no significant changes were observed at TF compared to T0 (Table 3). At TF, the BBM did not significantly change by increasing the Fe amount (5, 10, and 20 mg L^{-1}) (Table 3). In STWW samples, the BBM value was 7.90 log_{10} CFU mL^{-1} on average at T0 and no significant changes were observed compared to TF (Table 3). Only the sample treated with Fe at 20 mg L^{-1} significantly increased at TF to 8.61 ± 0.15 log_{10} CFU mL^{-1}. These results are the consequence of the iron being in solution that can be easily assimilated at this step, and that helps the bacteria's resistance to PCP toxicity and contributes to its effective removal from the media.

In sterile STWW, increasing the rate of Fe leads to a better bacterial development, and the strain of *Ps. putida* by producing pyoverdine enhanced the effectiveness of PCP-polluted sites' bioremediation. This pyoverdine production is known as largely

been affected by the Fe availability in the medium. The addition of Fe in the medium regulates the siderophore production and the PCP transformation throughout bacterial activity. In addition, the chloride content is untimely related to PCP degradation.

According to literature, the majority of the iron available in the medium and in the bacterial cell would be bound to siderophores, which are small peptides capable of forming complexes – siderophores Fe^{3+} – or iron chelators. These siderophores are a form of iron transit and hogging and a means of internalizing iron into the cell, which is necessary for its functioning; therefore, siderophores are well regarded as an important form of antagonism and rivalry between organisms living in the environment (Vaulont & Schalk 2015). Among the molecules synthesized by bacterial microorganisms and especially of the genus *Pseudomonas*, pyoverdines, fluorescent pigments, are fluorescent siderophores and an antibiotic oligopeptide produced in particular by *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, and are well considered as virulence and invasion factors of *Pseudomonas* (Mehri *et al.* 2011, 2012). So, bacteria can assimilate iron as an essential element for nutrient metabolism under aerobic condition (Beasley *et al.* 2019). In overall, iron is not soluble in liquid media which makes difficult its assimilation. As reported by Chen *et al.* (2011), iron can be chelated by PCP in the biological environment and become available for the metabolism and development of bacteria. The work of Chen *et al.* (2018) showed an interaction between PCP and Fe during degradation experiments related to some radical cations.

3.4.3. Inoculum volume

The result of inoculum volume variations of 0.5, 1, and 2 mL of the *Ps. putida* AE015451, at a PCP rate of 30 mg L^{-1} in the MSM medium and sterile STWW, is reported in Table 3. The variation of the inoculum volume directly affected the BBM content in the MSM medium. Both at T0 and TF and by increasing the inoculum, the BBM showed a significant increase (Table 3). Besides, by comparing the two incubation periods, T0 and TF, the BBM showed a significant increase following the application of 0.5 and 1 mL of inoculum (Table 3). Contrariwise in the case of the STWW experiment, no significant changes in BBM are observed in both T0 with on average 7.87 $\log_{10}$ CFU mL^{-1}, and TF with on average 8.25 $\log_{10}$ CFU mL^{-1} (Table 3).

3.4.4. pH variation

Table 3 shows the effect of 4, 5, 6.3, 7, and 8 pH variations on the BBM content in MSM and STWW, respectively, at a PCP rate of 30 mg L^{-1} at T0 and after 7 days. In the STWW medium, no significant changes in BBM content are registered at T0 and TF (Table 3). As for the MSM and at T0, a significant increase of BBM is registered, from 3.66 ± 0.92 $\log_{10}$ CFU mL^{-1} at pH 4 to 7.91 ± 1.03 $\log_{10}$ CFU mL^{-1} at pH 8 (Table 3). Equally and at TF, the BBM showed an increase in the MSM at pH 5 and 6.3 with values of 8.70 ± 0.92 and 9.24 ± 0.35 $\log_{10}$ CFU mL^{-1}, respectively (Table 3). By increasing the pH to 7 and 8, the BBM showed a significant decrease as compared to the case of T0. Thus, the pH showed an influence on the microbial growth and the removal and biotransformation process of PCP. These results obtained could be explained by the importance of the pH in protecting the bacteria from stresses, as reported by Zarkan *et al.* (2019).

3.5. Pyoverdine production

The ability of *Ps. putida* strain AE015451 to produce PVD is detected by a spectrophotometric technique in CAA medium with or without PCP (30 and 50 mg L^{-1}) and Fe (5, 10, and 20 mg L^{-1}) (Figure 2). We have monitored, respectively, the PVD production at different pHs 5, 6.3, and 8.

3.5.1. Pyoverdine production at pH 5.0

When 1 and 2 mL of inoculum are introduced in the CAA without PCP and at pH 5, the PVD content is revealed as important with 6.88 and 6.39×10^{-8} mol mL^{-1}, respectively (Figure 2(a)). Besides, the PVD content showed a decrease if the PCP increased from 30 to 50 mg L^{-1}. As well, no significant changes in PVD production at 30 mg L^{-1} of PCP following the inoculum volume increase, whereas the PVD showed an increase after the inoculum increase, especially to 1 mL and with 5.38×10^{-8} µmol mL^{-1} (Figure 2(a)). The addition of iron caused a net decrease in PVD production (CAA + Fe treatment; Figure 2(a)). The addition of Fe in CAA + PCP 30 and especially at 50 mg L^{-1} showed a net development of the pyoverdine production. In the case of CAA + Fe + PCP 30 mg L^{-1}, a significant decrease in PVD is observed because of the Fe increase. In addition, the PVD production appeared not related to the inoculum volume, since at 5 and 10 mg L^{-1} of Fe + 50 mg L^{-1} PCP no significant changes are observed after the inoculum volume increase. Contrariwise, in the CAA + Fe + PCP 50 mg L^{-1}, a different pattern of behavior is seen. The PVD production reached the value of the control without PCP after adding 1 mL of inoculum showing 7.20×10^{-8} µmol mL^{-1} (Figure 2(a)). These samples are characterized by a black precipitate at the bottom of the reaction tube.

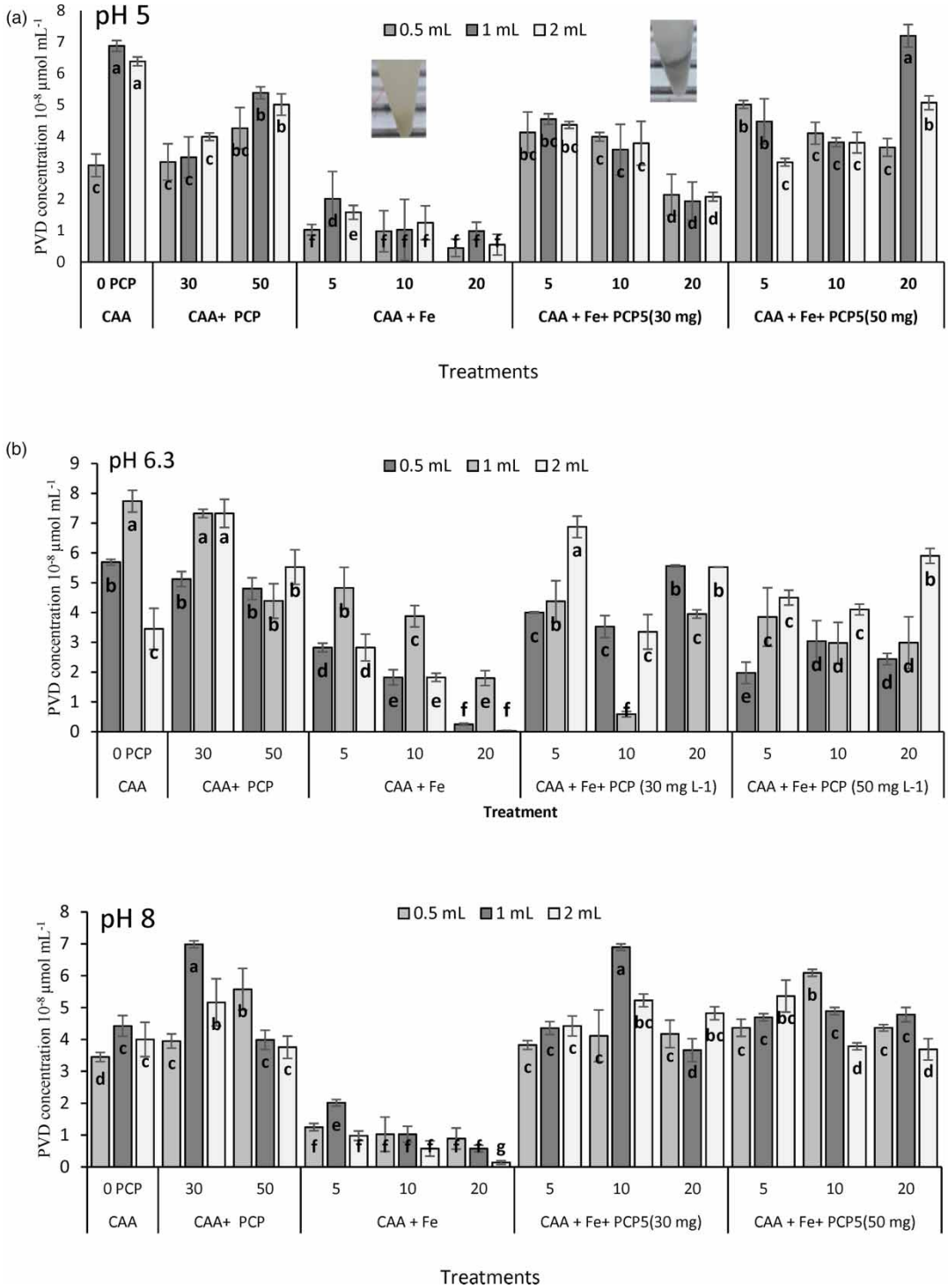


Figure 2 | Pyoverdine (PVD) production at pH 5, 6.3, and 8 according to PCP and iron addition in the medium. CAA, Casamino-acid medium; Fe, iron; PCP, pentachlorophenol. Different lowercase letters show significant differences among treatment according to Duncan *post-hoc* tests ($P < 0.01$).

3.5.2. Pyoverdine production at pH 6.3

The PVD production at pH 6.3 is also monitored and the result is reported in Figure 2(b). In the control experiment of CAA without PCP, the better PVD production was observed by adding 1 mL of the inoculum. In the experiment of CAA + PCP

30 mg L^{-1}, the PVD production reached the value registered for the control experiment without PCP after adding 1 or 2 mL of inoculum, with 7.32×10^{-8} µmol mL^{-1} (Figure 2(b)). The addition of Fe at 5, 10, and 20 mg L^{-1} allowed a reduction of the PVD production, but in the experiment inoculated with 1 mL of *Ps. putida*, *we* registered the larger value within this treatment with 4.85, 3.87, and 1.80×10^{-8} µmol mL^{-1} in CAA + 5, 10, or 20 mg L^{-1} of Fe, respectively (Figure 2(b)). Furthermore, CAA + Fe + PCP 30 mg L^{-1} showed the larger value of PVD production (6.87×10^{-8} µmol mL^{-1}) as compared to the control without PCP, and with 5 mg L^{-1} of Fe. In CAA + Fe + PCP 50 mg L^{-1}, no further changes compared to the control are observed about the PVD production (Figure 2(b)).

3.5.3. Pyoverdine production at pH 8.0

At pH 8, the better PVD production is achieved in the CAA supplemented with 30 mg L^{-1} of PCP, with 6.98×10^{-8} µmol mL^{-1}, and in the CAA + Fe 10 mg L^{-1} + PCP 30 mg L^{-1} with 6.89×10^{-8} µmol mL^{-1} (Figure 2(c)). The control experiment without PCP registered a decrease in PVD production as compared to the one recorded at pH 5 and 6.3 (Figure 2(a)–2(c)). Similar to the experiment at pH 5, the addition of Fe at 5, 10, and 20 mg L^{-1} resulted in a decrease in the PVD production. No further changes registered in PVD production in the CAA + Fe (5, 10, and 20 mg L^{-1}) + PCP at 30 and 50 mg L^{-1} (Figure 2(c)).

In contrast to the results obtained with PCP, the iron addition in the CAA does not affect the production of PVD. These most recent results can be attributed to the PVD's positive effect, which was carried out after PCP adaptation or neutralization by adsorption, conditioning the bacteria's tolerance to the PCP toxic effect.

This result followed Werheni Ammeri *et al.* (2016) and Karan *et al.* (2010) findings that showed *Ps. putida* to be more active at acidic pH for PCP removal. So, the PVD production was promoted in CAA and at pH 5. This result did not agree with the one of Mehri *et al.* (2011), who registered the best PVD production at pH 6.3.

3.6. Biofilm formation

The biofilm formation was tested for different concentrations of PCP (0, 30, 100, and 300 mg L^{-1}) and at different iron rates (5, 10, and 20 mg L^{-1}) in MSM and Brain heart infusion (BHI) medium. The BHI medium known as a good nutrient-rich medium used to culture a variety of fastidious organisms, and by increasing the PCP rates in the medium have allowed a slight decrease in the biofilm formation from 3.54 to 2.96 in the BHI without PCP and with PCP at 300 mg L^{-1}, respectively (Figure 3). In the MSM medium, the larger biofilm formation of 2.80 is observed in the experiment with PCP at 30 mg L^{-1}. In the experiment using PCP at 30 mg L^{-1}, a larger biofilm development of 2.80 is seen in the MSM medium. By increasing the

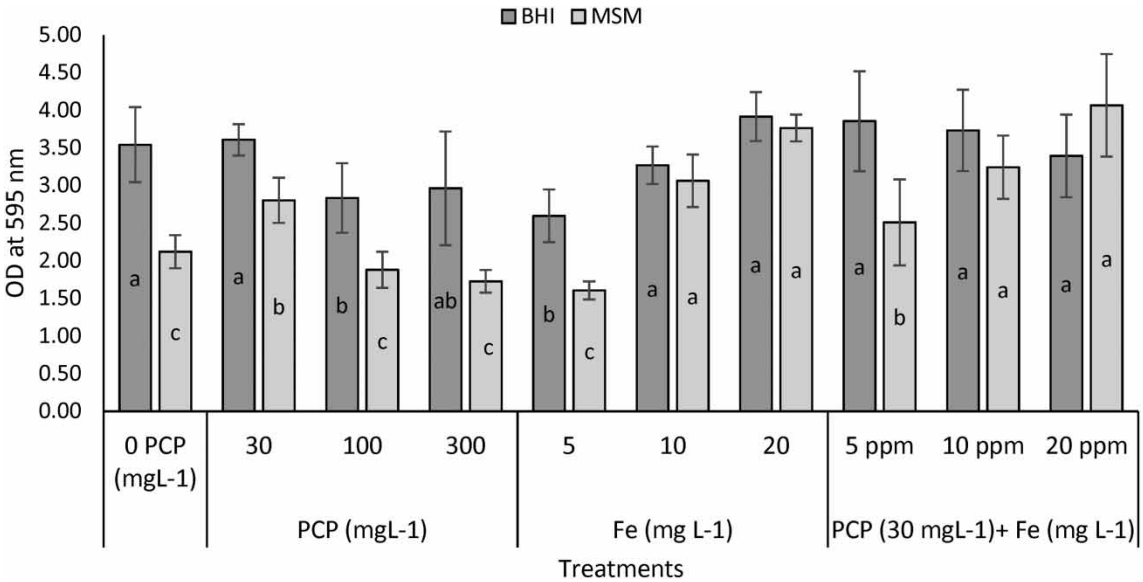


Figure 3 | Biofilm formation in the BHI and MSM medium supplemented or without PCP by the strain *Ps. putida* AE015451 at 30 °C after 48 h. Different lower letters show significant differences among treatments, in the same sampling time at the Duncan *post-hoc* test ($P < 0.05$). Fe, iron; PCP, pentachlorophenol.

PCP rates, the biofilm development return to the value registered for the control (Figure 3). In the BHI and MSM medium and by adding only Fe at 5, 10, and 20 mg L^{-1}, a significant increase in the biofilm production was observed with 3.92 and 3.76 with Fe 20 mg L^{-1} in BHI and MSM, respectively (Figure 3). The grouping of the iron at 5, 10, and 20 mg L^{-1} and the PCP rate of 30 mg L^{-1} showed no change in the biofilm production in the BHI growth medium; but in the MSM growth medium, a significant biofilm development was observed according to the rate of Fe increase (Figure 3). Thus, the increase in toxicity marked by the PCP had enhanced the development of bacterial biofilm. Biofilm systems have been demonstrated as effective in removing toxic compounds from wastewater according to various electrostatic and hydrophobic interactions and adsorption/desorption phenomena (Rahman & Anuar 2009). Also, several studies have shown bacterial biofilm development as a behavioral response to survival and success when bacterial cells were exposed to various environmental stresses during the *in-situ* PCP bioremediation (Amaya-Chavez *et al.* 2006). These results are comparable to those reported by several studies on *P. aeruginosa* biofilm such as the one of Filloux & Vallet (2003).

Our research supports Saygin & Baysal (2020)'s findings that PCP and its different residual metabolites should encourage the creation of microbial biofilm to protect against harmful substances. Many researches have advocated a near affiliation among hydrocarbon removal and microbial biofilm development (Verhagen *et al.* 2011; Dasgupta *et al.* 2013; Meliani & Ben Soltane 2014). Besides, the study of Amaya-Chavez *et al.* (2006) and Saraswathy *et al.* (2001) confirmed that the microbial biofilm development represented a behavioral reaction to survival and success while microbial cells have been exposed *in situ* to diverse environmental constraints. To conclude, our main results showed that *Pseudomonas* and its siderophore producing pyoverdine contribute to the PCP removal in the presence of iron.

3.7. Contour plot analysis and response-specific surface

Figures 4 and 5 show the contour plot of bacterial biomass. This last approach allowed to see how a response variable like pH, chloride, PCP rates, iron rates is related to the bacterial biomass. The contour plot showed the relationship between

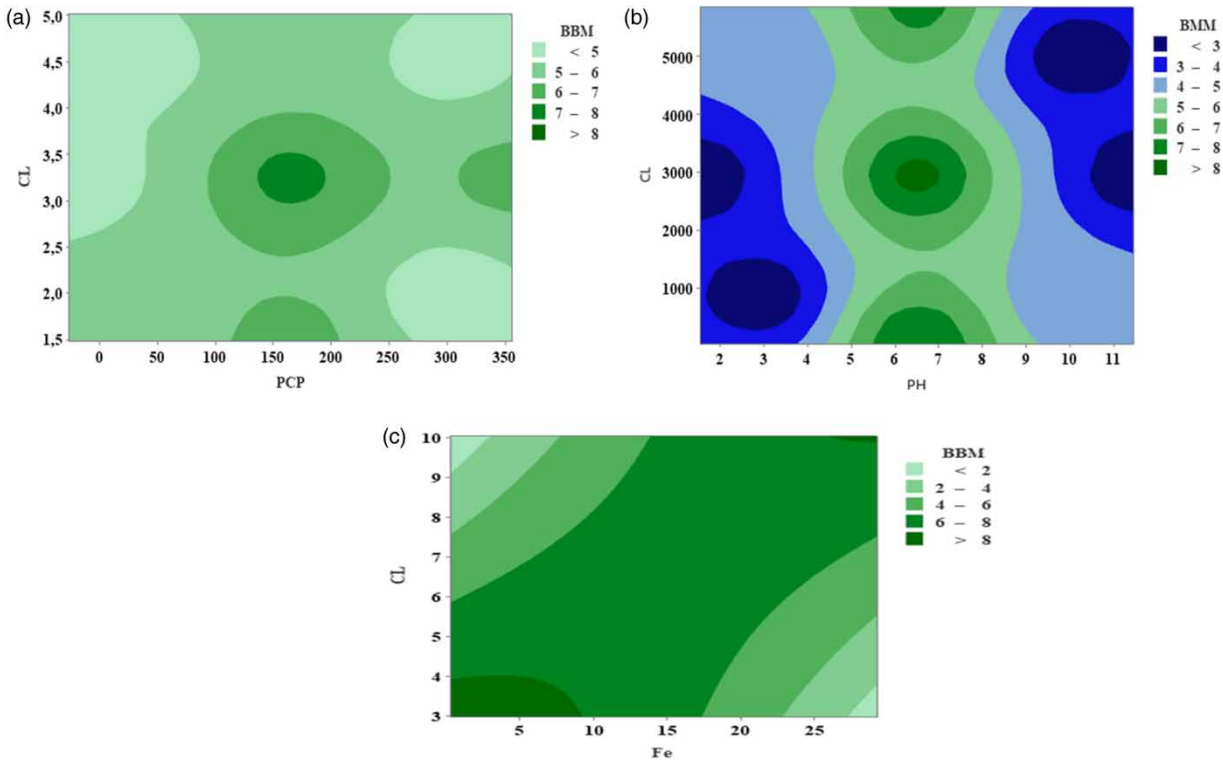


Figure 4 | (a) Contour plot of bacterial biomass (log CFU mL^{-1} medium) vs. chloride, PCP rates; (b) contour plot of bacterial biomass (log CFU mL^{-1} medium) vs. chloride, pH; and (c) contour plot of bacterial biomass (log CFU mL^{-1} medium) vs. chloride, iron (Fe) in MSM.

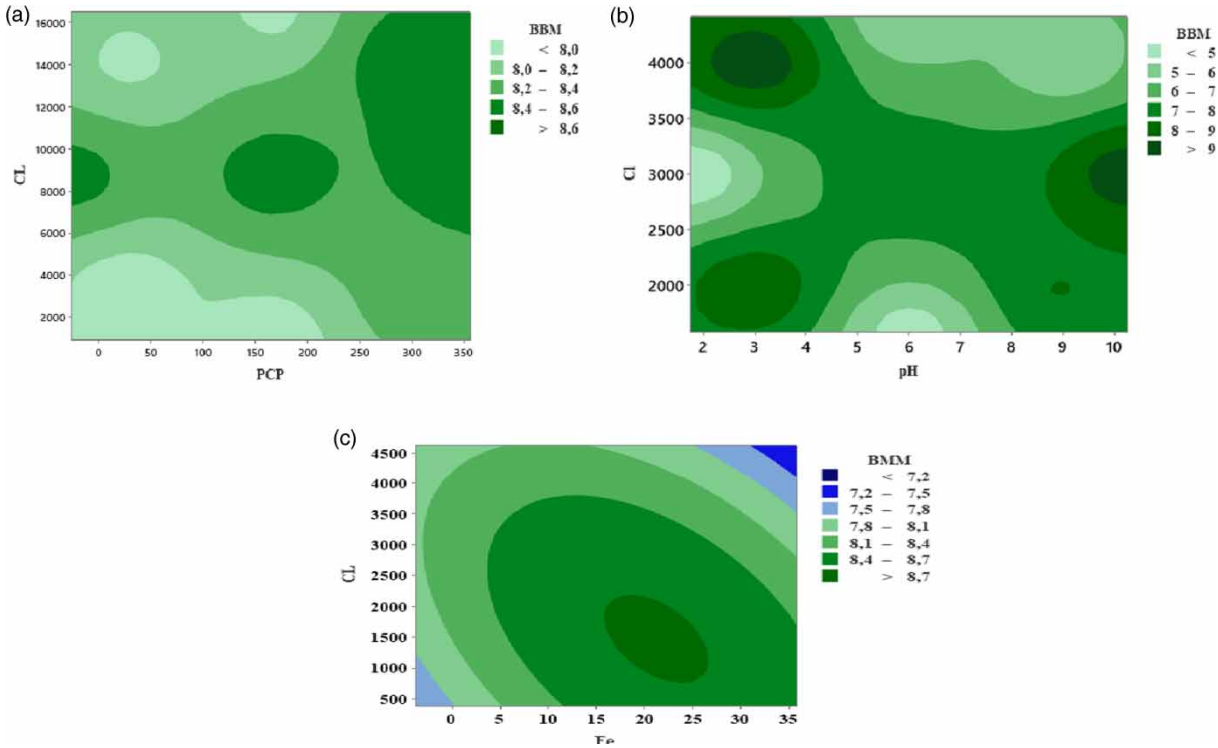


Figure 5 | (a) Contour plot of bacterial biomass (log CFU mL^{-1} medium) vs. chloride, PCP rates; (b) contour plot of bacterial biomass (log CFU mL^{-1} medium) vs. chloride, pH; and (c) contour plot of bacterial biomass (log CFU mL^{-1} medium) vs. chloride, iron (Fe) in TSWW.

chloride and PCP rates, or Fe or pH, in bacterial biomass. Darker regions showed the high intensity of bacterial biomass development of *Ps. putida* AE015451. These high responses seemed to form a ridge running from the upper middle to the lower right of the graph. The contour plot proves that, in liquid MSM the higher bacterial biomass was registered at a PCP contamination rate from 100 to 200 mg L^{-1}, pH at the range of 6–7 and Fe rates 5–10 mg L^{-1}. In STWW, the higher bacterial biomass appeared at the PCP range of 200–300 mg L^{-1}, pH at the range of 2–5 mg L^{-1} and Fe rates of 15–25 mg L^{-1}. These results confirmed that the strain *Ps. putida* AE015451 can tolerate the PCP toxicity in STWW more than MSM.

The effects of two independent variables such as PCP and Fe rates showed the maximum responses to 200 mg L^{-1} PCP and 20 mg L^{-1} Fe (Figure 4(a) and 4(b)).

Figure 6(a) and 6(b) of the response surface plot in BHI and in MSM medium shows an important interaction effect between biofilm formation, PCP rates (mg L^{-1}), and Fe rates (mg L^{-1}) in the bioaugmentation process. Increasing the Fe and PCP rates resulted in higher biofilm development. Therefore, the PCP–Fe interaction helps the bacteria to tolerate and grow within toxic and hostile biotopes.

4. CONCLUSION

The bioaugmentation of a secondary wastewater treatment process adopted in this study for PCP removal appeared to be an efficient alternative, less expensive, more extensive and more environmentally friendly tool as compared to the conventional physico-chemical methods. This bio-process showed high PCP removal for *Ps. putida* AE015451 and at 100 mg L^{-1}. After 72 h of incubation in STWW, this selected *Pseudomonas* strain removed about 90% of PCP. So, this strain of *Ps. putida* AE015451 could play an important role in the bioaugmentation process associated with its PGPR properties. Thus, it can help macrophytes to tolerate the toxicity of some compounds such as PCP and other insecticides, and enhance their decontamination properties by secreting PVD. The effect of adding iron as $FeSO_4$ showed this latter could react with PCP, forming

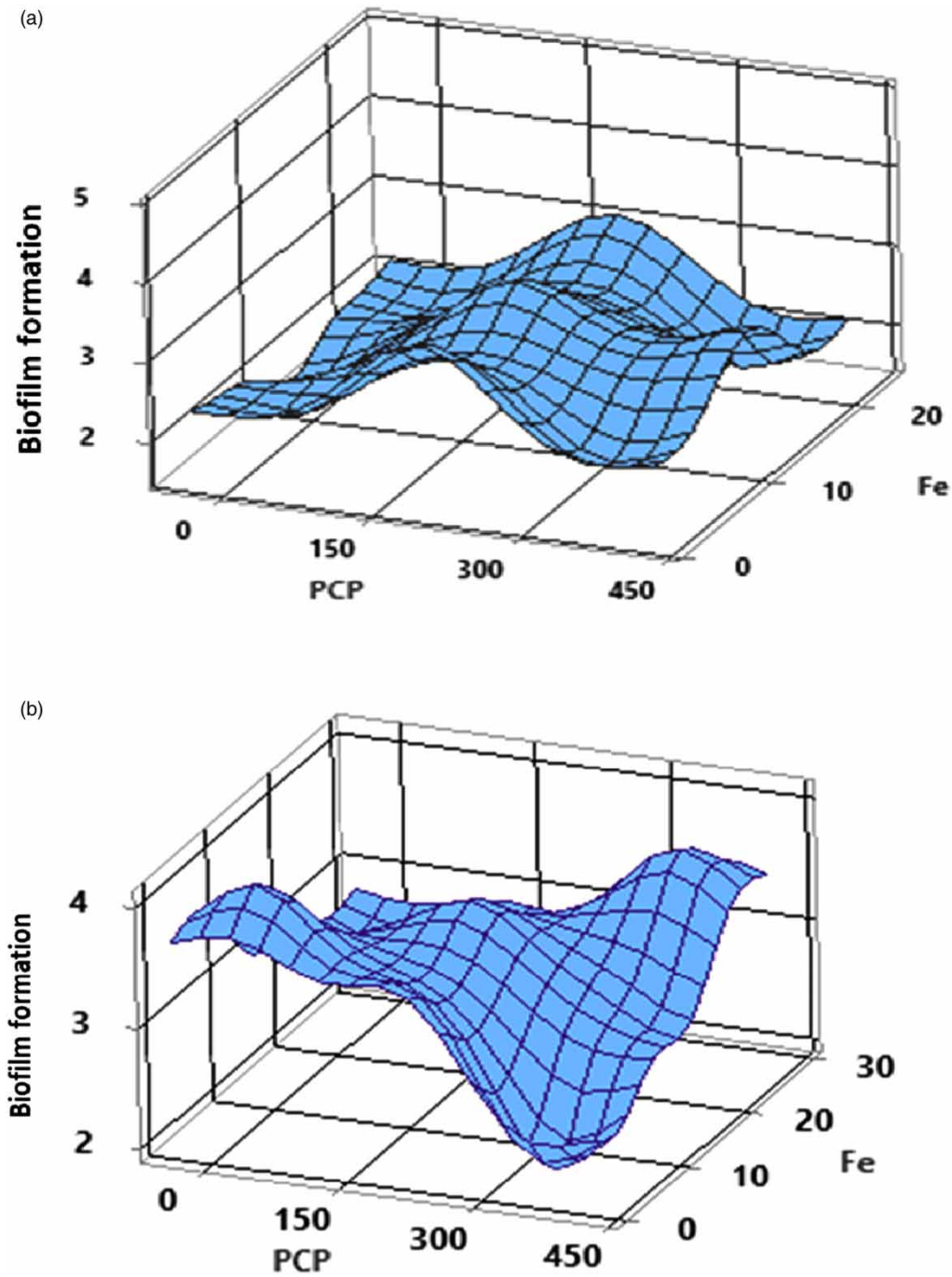


Figure 6 | Response surface plot (a) in BHI medium and (b) in MSM of interaction effects between biofilm formation, PCP concentration (mg L^{-1}), and iron rates (mg L^{-1}).

neutral Fe–PCP complexes, which contribute to the development and growth of bacteria, and directly the pollution reduction. The ability of bacteria to form condensed biofilm enhances their degradative capacity and resistance to xenobiotic compounds such as PCP used as a carbon source. Our study demonstrates the effectiveness of PCP bio-removal by adding bacteria that can use the pollutant as a nutrient source. Further research is needed to extend these experiments to wastewater to develop bacterial weathering-based bioremediation processes.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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